PCT/SE00/00223

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 09 November 2001 (09.11.01)	
International application No. PCT/SE00/00223	**Applicant's or agent's file reference** P04756PC00
International filing date (day/month/year) 04 February 2000 (04.02.00)	**Priority date** (day/month/year) 05 February 1999 (05.02.99)
Applicant KORSGREN, Olle et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

05 September 2000 (05.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

CORRECTED

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer François BAECHLER
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 October 2000 (11.10.00)	
International application No. PCT/SE00/00223	**Applicant's or agent's file reference** P04756PC00
International filing date (day/month/year) 04 February 2000 (04.02.00)	**Priority date** (day/month/year) 05 February 1999 (05.02.99)
Applicant KORSGREN, Olle et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 September 2000 (07.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [ ] was

[X] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. E. Stoffel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00223

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/36, A61K 38/55, C12N 5/06 // C07K 14/745
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 19623440 A1 (WAGNER, KARL-HEINZ, DR. MED.), 18 December 1997 (18.12.97) --	1-11
X	Transplantation Proceedings, Volume 28, No 3, June 1996, Y. Nomura, S. Ito, "Unpurified Islet Cell Transplantation in Diabetic Rats" page 1849 - page 1850 --	1-11
X	Transplantation Proceedings, Volume 25, No 4, August 1993, U.T. Hopt et al, "Prevention of Early Postoperative Graft Thrombosis in Pancreatic Transplantation" page 2607 - page 2608	1-8,10-11
Y	--	9

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	08 -06- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00223

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Transplantation Proceedings, Volume 20, No 3, June 1988, J. Tollemar et al, "Anticoagulation Therapyfor Prevention of Pancreatic Graft Thromb osis: Benefits and Risks" page 479 - page 480	1-8,10-11
Y		9
	--	
X	Dialog Information Services, File 73, EMBASE, Dialog accession no. 04002091, Embase accession no. 1989171087, Rigotti P. et al: "Use of defibrotide in preventing vascular thrombosis in experimental pancreas transplantation"; & Surgical Research Communications (SURG. RES. COMMUN.) (United Kingdom) 1989, 6/2 (123-130)	1-8,10-11
Y		9
	--	
Y	WO 9105855 A1 (IMUTRAN LIMITED), 2 May 1991 (02.05.91)	9
	--	
X	Dialog Information Services, File 155,MEDLINE, Dialog accession no. 08102103, Medline accession no. 95134104, Tatarkiewicz K et al: "In vitro and in vivo evalutation of protamine-heparin membrane for microencapsulation of rat Langerhans islets"; & Artif Organs (UNITED STATES) Oct 1994, 18 (10) p736-9, ISSN 0160-564X	10
A		4
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PATENT COOPERATION TREATY

PCT

REC'D 28 JUN 2001
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P04756PC00/UA	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/SE00/00223	International filing date *(day/month/year)* 04.02.2000	Priority date *(day/month/year)* 05.02.1999
International Patent Classification (IPC) or national classification and IPC7 A 61 K 38/36, A 61 K 38/55, C 12 N 5/06 // C 07 K 14/745		
Applicant Corline Systems AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07.09.2000	06.06.2001
Name and mailing address of the IPEA/SE [illegible] Tel: [illegible] Fax: [illegible] Facsimile No. 08-667 72 88	Authorized officer Carolina Palmcrantz/EÖ Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/00223

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☐ the description:

pages _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

☐ the claims:

pages _____, as originally filed

pages _____. as amended (together with any statement) under article 19

pages _____, filed with the demand

pages _____, filed with the letter of _____

☐ the drawings:

pages _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

☐ the sequence listing part of the description:

pages _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/00223

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 11

because:

☒ the said international application, or the said claims Nos. 11
relate to the following subject matter which does not require an international preliminary examination *(specify)*:

See PCT Rule 67.1(iv): Methods for treatment of the human or animal body by surgery or therapy.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos.
are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	4-7, 9-10	YES
	Claims	1-3, 8	NO
Inventive step (IS)	Claims	4, 10	YES
	Claims	5-7, 9	NO
Industrial applicability (IA)	Claims	1-10	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The present application pertains to the use of a clotting preventing agent in the production of a drug for administration in connection with transplantation of insulin producing cells in the form of **isolated islets** to patients with insulin dependent diabetes mellitus (IDDM). The application further concerns isolated cells comprising islets of Langerhan´s which have been coated with a heparin conjugate on the islet surface.

The present invention is based on the finding that in autologous islet transplantation, the islets are rapidly coated by a layer of platelets which develops into a thrombus. By adding a clotting preventing agent in connection with the transplantation, thrombus formation is reduced.

The wordings "a clotting preventing agent" of claim 1, "an inhibitor of platelet activation" of claim 5 and "an inhibitor of complement" of claim 9, each covers an unknown number of structurally different compounds in addition to those mentioned in the application. It is not considered to be clear which compounds that are intended to be covered by the definitions. Therefore, the international search has been incomplete and restricted to those compounds exemplified in the application. Consequently, this report is based only on those compounds covered by the international search.

The international search report revealed seven documents of interest:

D1) DE 19623440 A1 (WAGNER, KARL-HEINZ, DR. MED.), 18 December 1997 (18.12.97)

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

D2) Transplantation Proceedings, Volume 28, No 3, June 1996, Y. Nomura, S. Ito. "Unpurified Islet Cell Transplantation in Diabetic Rats" page 1849 - page 1850

D3) Transplantation Proceedings, Volume 25, No 4, August 1993, U.T. Hopt et al, "Prevention of Early Postoperative Graft Thrombosis in Pancreatic Transplantation" page 2607 - page 2608

D4) Transplantetion Proceedings, Volume 20, No 3, JUNE 1988, J. Tollemar et al, "Anticoagulation Therapyfor Prevention of Pancreatic Graft Thromb osis: Benefits and Risks" page 479 - page 480

D5) Dialog Information Services, File 73, EMBASE, Dialog accession no. 04002091, Embase accession no. 1989171087, Rigotti P. Et al: "Use of defibrotide in preventing vasculr thrombosis in experimental pancreas transplantation"; & Surgical Research Communications (SURG. RES.COMMUN.)(United Kingdom) 1989, &72 (123-130)

D6) WO 9105855 A1 (IMUTRAN LIMITED) 2 May 1991 (02.05.91)

D7) Dialog Information Services, file 155, MEDLINE, Dialog accession no. 08102103, Medline accession no. 95134104, Tatarkiewicz K et al: "In vitro and in vivo evalutation of protamine-heparin membrane for microencapsulation of rat Lagerhans islets"; & Artif Organs (UNITED STATES) Oct 1994, 18 (10) P 736-9M ISSN 0160-564X

D1 concerns the use of clotting preventing agents, e.g. heparin, in connection with transplantation of insulin producing cells such as islets of Langerhans (see claim 8). The cells may be in the form of microencapsulated islets (see figure 1 and claim 10).

D2 discloses the transplantation of unpurified pancreatic cells in diabetic rats. The transplantation is performed with anticoagulants (see section *Material and Methods*).

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

D3-D5 concern the prevention of postoperative graft thrombosis with anticoagulants in pancreatic transplantation.

Although not explicitly mentioned in the cited documents it is considered to be obvious to a person skilled in the art to use one or more known clotting preventing agents in addition to heparin, such as an inhibitor of platelet activation.

D6 discloses a method of transplanting animal tissue, e.g. islets of Langerhans, into a recipient, wherein the tissue is derived from a donor of a different species from the recipient. The method involves grafting the tissue into the recipient and providing in association with the grafted tissue one or more hompologous complement restriction factors to prevent the complete activation of complement (see claims 1-4). Therefore, in view of D6 it is considered to be obvious to a person skilled in the art to also use an inhibitor of complement in connection with the transplantation.

D7 concerns the in vitro and in vivo evaluation of protamine-heparin membrane for microencapsulation of rat Langerhans islets.

Thus, it is considered to be well-known in the prior art to use a clotting preventing agent, e.g. heparin, in connection with transplantation of insulin producing cells such as islets of Langerhan´s. However, the cells according to the cited prior art documents are transplanted as microencapsulated cells or as pancreatic tissue, that is, none of the documents concerns the transplantation of isolated islets in the meaning of not being artificially encapsulated or being absent of exocrine pancreatic tissue. The problem with thrombus formation in connection with transplantation of isolated islets has not been identified in either of D1-D7. Therefore, in view of the cited prior art documents, it is considered to be non-obvious to a person skilled in the art that the efficiency of transplantation of isolated islets would be improved when administrated together with a clotting preventing agent. However, the wording "in the form of isolated islets" in claim 1 is not considered to clearly exlude

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

the microencapsulated islets described in D1, which also are considered to be "isolated". Therefore, with the present wording claims 1-3 and 8 are not considered to be novel and consequently claims 5-7 and 9 are not considered to involve an inventive step, whereas claims 4 and 10 are considered to fulfil the requirements of novelty, inventive step and industrial applicability.

09/890936

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P04756PC00/UA		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SE00/00223	04.02.2000	05.02.1999
International Patent Classification (IPC) or national classification and IPC A 61 K 38/36, A 61 K 38/55, C 12 N 5/06 // C 07 K 14/745		
Applicant Corline Systems AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

CORRECTED VERSION

Date of submission of the demand	Date of completion of this report
05 SEP 2000	06.06.2001
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88	Authorized officer Carolina Palmcrantz/EÖ Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

CORRECTED

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/00223

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☐ the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the claims:
pages _____, as originally filed
pages _____, as amended (together with any statement) under article 19
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the drawings:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the sequence listing part of the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/00223

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 11

because:

☒ the said international application, or the said claims Nos. 11
relate to the following subject matter which does not require an international preliminary examination *(specify)*:

See PCT Rule 67.1(iv): Methods for treatment of the human or animal body by surgery or therapy.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _____
are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. _____ are so inadequately supported
by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/00223

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	4-7, 9-10	YES
	Claims	1-3, 8	NO
Inventive step (IS)	Claims	4, 10	YES
	Claims	5-7, 9	NO
Industrial applicability (IA)	Claims	1-10	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The present application pertains to the use of a clotting preventing agent in the production of a drug for administration in connection with transplantation of insulin producing cells in the form of **isolated islets** to patients with insulin dependent diabetes mellitus (IDDM). The application further concerns isolated cells comprising islets of Langerhan´s which have been coated with a heparin conjugate on the islet surface.

The present invention is based on the finding that in autologous islet transplantation, the islets are rapidly coated by a layer of platelets which develops into a thrombus. By adding a clotting preventing agent in connection with the transplantation, thrombus formation is reduced.

The wordings "a clotting preventing agent" of claim 1, "an inhibitor of platelet activation" of claim 5 and "an inhibitor of complement" of claim 9, each covers an unknown number of structurally different compounds in addition to those mentioned in the application. It is not considered to be clear which compounds that are intended to be covered by the definitions. Therefore, the international search has been incomplete and restricted to those compounds exemplified in the application. Consequently, this report is based only on those compounds covered by the international search.

The international search report revealed seven documents of interest:

D1) DE 19623440 A1 (WAGNER, KARL-HEINZ, DR. MED.), 18 December 1997 (18.12.97)

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

D2) Transplantation Proceedings, Volume 28, No 3, June 1996, Y. Nomura, S. Ito. "Unpurified Islet Cell Transplantation in Diabetic Rats" page 1849 - page 1850

D3) Transplantation Proceedings, Volume 25, No 4, August 1993, U.T. Hopt et al, "Prevention of Early Postoperative Graft Thrombosis in Pancreatic Transplantation" page 2607 - page 2608

D4) Transplantetion Proceedings, Volume 20, No 3, JUNE 1988, J. Tollemar et al, "Anticoagulation Therapyfor Prevention of Pancreatic Graft Thromb osis: Benefits and Risks" page 479 - page 480

D5) Dialog Information Services, File 73, EMBASE, Dialog accession no. 04002091, Embase accession no. 1989171087, Rigotti P. Et al: "Use of defibrotide in preventing vasculr thrombosis in experimental pancreas transplantation"; & Surgical Research Communications (SURG. RES.COMMUN.)(United Kingdom) 1989, &72 (123-130)

D6) WO 9105855 A1 (IMUTRAN LIMITED) 2 May 1991 (02.05.91)

D7) Dialog Information Services, file 155, MEDLINE, Dialog accession no. 08102103, Medline accession no. 95134104, Tatarkiewicz K et al: "In vitro and in vivo evalutation of protamine-heparin membrane for microencapsulation of rat Lagerhans islets"; & Artif Organs (UNITED STATES) Oct 1994, 18 (10) P 736-9M ISSN 0160-564X

D1 concerns the use of clotting preventing agents, e.g. heparin, in connection with transplantation of insulin producing cells such as islets of Langerhans (see claim 8). The cells may be in the form of microencapsulated islets (see figure 1 and claim 10).

D2 discloses the transplantation of unpurified pancreatic cells in diabetic rats. The transplantation is performed with anticoagulants (see section *Material and Methods*).

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

D3-D5 concern the prevention of postoperative graft thrombosis with anticoagulants in pancreatic transplantation.

Although not explicitly mentioned in the cited documents it is considered to be obvious to a person skilled in the art to use one or more known clotting preventing agents in addition to heparin, such as an inhibitor of platelet activation.

D6 discloses a method of transplanting animal tissue, e.g. islets of Langerhans, into a recipient, wherein the tissue is derived from a donor of a different species from the recipient. The method involves grafting the tissue into the recipient and providing in association with the grafted tissue one or more hompologous complement restriction factors to prevent the complete activation of complement (see claims 1-4). Therefore, in view of D6 it is considered to be obvious to a person skilled in the art to also use an inhibitor of complement in connection with the transplantation.

D7 concerns the in vitro and in vivo evaluation of protamine-heparin membrane for microencapsulation of rat Langerhans islets.

Thus, it is considered to be well-known in the prior art to use a clotting preventing agent, e.g. heparin, in connection with transplantation of insulin producing cells such as islets of Langerhan´s. However, the cells according to the cited prior art documents are transplanted as microencapsulated cells or as pancreatic tissue, that is, none of the documents concerns the transplantation of isolated islets in the meaning of not being artificially encapsulated or being absent of exocrine pancreatic tissue. The problem with thrombus formation in connection with transplantation of isolated islets has not been identified in either of D1-D7. Therefore, in view of the cited prior art documents, it is considered to be non-obvious to a person skilled in the art that the efficiency of transplantation of isolated islets would be improved when administrated together with a clotting preventing agent. However, the wording "in the form of isolated islets" in claim 1 is not considered to clearly exlude

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

the microencapsulated islets described in D1, which also are considered to be "isolated". Therefore, with the present wording claims 1-3 and 8 are not considered to be novel and consequently claims 5-7 and 9 are not considered to involve an inventive step, whereas claims 4 and 10 are considered to fulfil the requirements of novelty, inventive step and industrial applicability.

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A1

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(54) Title: NOVEL USE WITHIN TRANSPLANTATION SURGERY

(57) Abstract

The present invention is within the field of transplantation surgery. More closely, the present invention relates to use of a clotting preventing agent in the production of a drug for administration in association with transplantation of insulin producing cells in the form of isolated islets to patients with insulin dependent diabetes mellitus, IDDM. The invention is expected to significantly improve the clinical outcome of transplantation of islets of Langerhans.

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Title: Novel use within transplantation surg ry

Field of the invention

The present invention is within the field of transplantation surgery. More closely, the present invention relates to use of a clotting preventing agent in the production of a drug for administration in association with transplantation of cells and tissue, such as insulin producing cells to patients with insulin dependent diabetes mellitus, IDDM.

Background of the invention

The only option to achieve permanent normoglycemia in diabetic patients is a renewal of the β-cells, either by transplantation of segmental/whole pancreas or isolated islets of Langerhans. Transplantation of isolated islets is considerably less successful compared to whole pancreas transplantation. The immunological barrier, the underlying autoimmune disease and the immunosuppressive drugs used, are the same in both types of transplantation. Thus, there is no obvious immunological explanation as to why transplantation of whole pancreas is more successful than islet transplantation.

If, however, the problems related to the unsuccessful outcome of transplantation of islets were identified and a technical and practical solution was developed, obvious benefits for the patients would be created implying interesting commercial opportunities.

The prior art in this field is largely confined to measures aiming at reducing immunological reactions. WO 9711607 describes transplantation of microencapsulated insulin producing cells as a means of protecting the cells from immunological reactions and/or combined with treating the recipient with a substance that would inhibit an immune-system costimulation. WO 9105855 describes transplantation of islets of animal origin and that the

animal tissue should be modified to contain homologous complement restriction factors. DE 19623 440 A1 describes methods for encapsulation of islets and points out that the artificial encapsulation material may induce platelet activation, coagulation and complement activation, and therefore the encapsulation material should be modified to allow release of inhibiting substances as e.g., heparin, hirudin or Marcumar. US 5 635 178 is not related to transplantation of islets but describes monoclonal antibodies having inhibitory activity towards the terminal complex of complement and that such antibodies can be used to reduce activation of platelets and endothelial cells.

It is evident for those skilled in the art that measures aiming at inhibiting immunological reactions in connection with transplantation of islets regardless of being allogenic or xenogenic have not lead to a satisfactory result in respect of clinical outcome.

Summary of the invention

The present inventors have performed experiments implying adding human, adult porcine or fetal porcine islets to human whole blood and have been struck by the vigorous coagulation occurring when these islets were injected into human ABO-compatible blood. As judged by microscopical examinations it is evident that the islets are rapidly coated by a layer of platelets which soon develops into an organised thrombus. This biological event has previously not been considered and is now suggested to be a major explanation as to why the outcome of autologous islet transplantation has been comparatively unsuccessful. The present invention is related to measures to reduce this incompatibility reaction that can either be directed towards inhibiting activation of platelets, mono- or polymorhonuclear cells or the enzyme cascade of coagulation. Regardless of the initiating event, any of these reactions will lead to generation of thrombin, which eventually converts fibrinogen to fibrin. The generation of thrombin can easily be monitored by measuring the thrombin- antithrombin complex (TAT complex).

Hence, the present invention is concerned with therapeutic measures to inhibit TAT complex formation upon exposure of allogenic or xenogenic islets to whole blood.

Therefore, the present invention relates to a use of a clotting preventing agent in the production of a drug for administration in connection with transplantation of cells and tissue, such as insulin producing cells in the form of isolated islets to patients with insulin dependent diabetes mellitus, IDDM.

Preferably, the clotting preventing agent is an anticoagulant, such as heparin or fractions or derivatives thereof. Alternatively, hirudin, oxalate, citrate etc. can be used.

In one embodiment of the invention, the islet cells are coated with heparin or fractions or derivatives thereof by preincubation of islets in a solution containing heparin or fractions or derivatives thereof. Using a conjugate of heparin to coat the islets, it was demonstrated that the modified islets had acquired an increased capacity to adsorb antithrombin and loop experiments (described below) demonstrated that it is possible to reduce clotting by using such modified islets.

In an alternative embodiment of the invention, the preventing agent is an inhibitor of platelet activation, such as a RGD (standard one letter code for amino acids) containing peptide or a monoclonal antibody which inhibits the interaction of platelet integrins with their specific ligands. This antibody is for example a monoclonal antibody or a peptide directed against the Fc receptor on platelets.

A combination of anticoagulant and inhibitor of platelet activation can be used as clotting preventing agent according to the invention or any other suitable combination of preventing agents. Optionally, the preventing agent(s) is/are supplemented by an inhibitor of complement.

Furthermore, the invention relates to a method for increasing survival of islet cells in connection with transplantation of insulin producing cells to patients with insulin dependent diabetes mellitus, IDDM, comprising prevention of clotting, monitored as reduced generation of thrombin-antitrombin complex.

Detailed description of the invention

The invention will be described more closely below in association with the accompanying drawings, in which

Fig. 1 is a graph showing percent aggregation of platelets following addition of islets to platelet rich plasma, PRP, as a function of time;

Fig. 2 shows a similar graph as in Fig. 1 but here a RGDS (standard one letter code for amino acids) tetrapeptide was added to PRP before islets were added; and

Fig. 3 shows a similar graph as in Figs. 1 and 2 but here a monoclonal antibody against the Fc receptor on platelets was added to PRP before islets were added.

All the in vitro experiments for studies of islets contacting whole blood were performed in a tubing loop model. The experimental model is a modification of a model for testing biomaterials that has previously been described (J. Clin. Immunol. 16, 223-230 (1996)). Tubings made of polyvinylchloride (PVC, i.d. = 6.3 mm, length = 300 mm) were modified with immobilized heparin according to a method developed by Corline Systems AB (Uppsala, Sweden) as disclosed in international patent application no WO93/05793. Briefly, the polymer surface is modified with a high molecular weight amine compound to add primary amine groups to the surface. A soluble conjugate prepared by covalent binding of approximately 60 mol of heparin per mol of a straight-chained polyallylamine is irreversibly bonded onto the amine surface of the tubings. This procedure results in a total surface

concentration of heparin of approximately 0.5 $\mu g/cm^2$. By using such heparin modified tubings it is possible to incubate the tubings with non-anticoagulated fresh human blood in a rocking device at 37°C for one hour with only moderate activation of blood (c.f. control column in Table 1 A and 1 B below). Unmodified tubings will invariably induce complete clotting at these experimental conditions. Addition of human islets or porcine adult or fetal islets lead to some remarkable observations. Complete clotting invariably occurred with a total loss of platelets, a sharp increase in the formation of TAT and a very significant increase in the markers of the early contact phase (FXIIa and FXIa) of coagulation (C.f. Table 1 A and 1 B). Histological examination revealed a dense layer of activated platelets immediately adjacent to the capsule of the islets.

The findings in vitro described above were confirmed in vivo by evaluation of porcine islets after intraportal transplantation in pigs. The porcine livers, removed 60 min. after islet transplantation, had a congested appearance with patchy dark discoloration's on the surface. In the portal veins blood clots were found, with a patchy adherence to the endothelium, and branching into the portal tree, partially occluding the vessels. The histological examination revealed islets entrapped in blood clots, with a disrupted islet morphology. Occasionally a fibrin tail could be observed extending away from the islet.

With reference to Table 1 B, it appears that the effect of adding an inhibitor of complement leads to reduced activation of complement, as expected, but there is no measurable effect on the clotting of blood or activation of platelets. If, however, soluble heparin was added to the experimental system there was a remarkable improvement in preservation of the number of platelets and reduced generation of TAT.

In another set of experiments the effects of inhibiting the interaction between platelet integrins and their specific ligands were investigated. With reference to Fig. 1-3, it appears that platelet aggregation is induced upon contact with

islets and that such aggregation can be prevented by blocking platelet integrins or Fc-receptors.

Porcine islets were surface modified by incubation in a buffered solution containing a high molecular weight conjugate of heparin (Corline Heparin Conjugate), as disclosed in WO 93/05793, and then rinsed by changing buffer several times. It was demonstrated that the modified islets had acquired an increased capacity to adsorb antithrombin and loop experiments showed that heparin modified islets resulted in reduced clotting compared to unmodified islets.

It is easily understood by those skilled in the art that there is a broad arsenal of agents that can be used to accomplish reduced clotting, and hence, the following non-limiting Examples are only used to demonstrate the principle behind the present invention.

Example 1: Effect of soluble heparin

Sixty ml of non-anticoagulated blood was collected from healthy blood donors using heparin-coated equipment. U-shaped tubings with a total volume of nine ml were filled with eight ml of blood immediately followed by addition of isolated human islets or porcine adult or porcine fetal islets (500 IEQ). The tubings were then closed into loops using connectors of titanium furnished with immobilised heparin. The tubing loops were placed vertically in a rocking device and the complete apparatus was placed in an incubator at 37°C for up to sixty minutes. At the end of the rocking period blood was collected in EDTA and the number of cells were counted in a automatic cell counter. The blood samples were then centrifuged at 4°C (3290xg, 20 min) and EDTA plasma was collected and immediately put at –70°C. Islets retrieved after blood perifusion were prepared for immunohistochemistry. The results are summarized in Table 1A and 1B below.

Table I A shows results of blood cell counts and coagulation and complement parameters before and after 60 min. of human islet perifusion with ABO-compatible fresh human blood or blood supplemented with heparin.

Table 1A

Table 1A: Blood cell counts and coagulation and complement parameters before and after 60 min. of human islet perifusion with ABO-compatible fresh human blood or blood supplemented with heparin.

	BEFORE	CONTROL	HUMAN ISLETS	
			WITHOUT ADDITIVES	HEPARIN
Platelets ($x10^9$)	233 ± 13.8	161.1± 9.3	5 ± 0.3***	114 ± 17*
Neutro. ($x10^9$)	3.23 ± 0.33	3.03 ± 0.32	0.83 ± 0.18***	2.56 ± 0.43
Mono. ($x10^9$)	0.36 ± 0.03	0.36 ± 0.04	0.03 ± 0.01***	0.28 ± 0.06
Lymph. ($x10^9$)	1.91 ± 0.12	1.77 ± 0.12	1.29 ± 0.12**	1.60 ± 0.20
C3a (ng/mL)	84 ± 4.7	507 ± 115	1259 ± 125.1***	565 ± 143.6
C5b-9 (AU/mL)	15.6 ± 2.9	95 ± 30	213 ± 43.4*	147 ± 39.6
FXIIa-AT (umol/L)	0.09 ± 0.01	0.36 ± 0.15	12.9 ± 0.9***	5.4 ± 1.7**
FXIa-AT (umol/L)	0.06 ± 0.01	0.12 ± 0.03	4.74 ± 0.48***	0.34 ± 0.12*
TAT (ug/mL)	12.5 ± 5.2	316 ± 100	20537 ± 1973***	4467 ± 2285

Control loops contained blood and culture medium (RPMI), but no islets. All values are stated as the Mean ± SE(M). TAT, Thrombin-antithrombin. The degree of significance is reported with respect to the controls.
(*$p<0.05$; **$p<0.01$; ***$p<0.001$; n.a. = not analysed).

Table I B shows results of blood cell counts and coagulation and complement parameters before and after 60 min. of adult and fetal porcine islet perifusion with fresh human blood or blood supplemented with the complement inhibitor C1 inactivator (C1-INA) or heparin.

Table 1B: Blood cell counts and coagulation and complement parameters before and after 60 min. of adult and fetal porcine islet perifusion with fresh human blood or blood supplemented with C1-INA or heparin.

	BEFORE	CONTROL	ADULT ISLETS			FETAL ISLETS
			WITHOUT ADDITIVE	C1-INA	HEPARIN	WITHOUT ADDITIVES
Platelets ($x10^9$)	237± 8.0	171 ± 9.0	4 ± 0.1***	4 ± 0	145 ± 13.0	4 ± 0***
Neutrophils ($x10^9$)	2.75 ± 0.21	2.52 ± 0.21	0.57 ± 0.07***	0.41 ± 0.13	3.00 ± 0.19	1.44 ± 0.17**
Monocytes ($x10^9$)	0.38 ± 0.02	0.37 ± 0.02	0.04 ± 0.01***	0.15 ± 0.01	0.35 ± 0.05	0.07 ± 0.01***
Lymphcytes ($x10^9$)	2.30 ± 0.14	2.13 ± 0.11	1.74 ± 0.10*	1.23 ± 0.38	1.88 ± 0.11	1.68 ± 0.25
C3a (ng/mL)	80.1± 7.3	545 ± 68	1435 ± 173***	1094 ± 78	486 ± 139	1601 ± 215***
C5b-9 (AU/mL)	15.8 ± 1.8	72 ± 10	283 ± 34***	183 ± 29	82 ± 22	302 ± 46***
FXIIa-AT (mmol/L)	0.18 ± 0.03	0.13 ± 0.00	8.96 ± 1.38***	19.65 ± 0.45	3.56 ± 1.60**	n.a.
FXIa-AT (mmol/L)	0.04 ± 0.00	0.03 ± 0.00	4.14 ± 0.48***	2.95 ± 0.15	0.53 ± 0.26	n.a.
TAT (ug/mL)	5.6 ± 1.1	139 ± 35	23886 ± 3494***	30250 ± 3450	505 ± 162***	34420 ± 4875

Control loops contained blood and culture medium (RPMI), but no islets. All values are stated as the Mean SE(M). TAT, Thrombin-antithrombin. The degree of significance is reported with respect to the controls.
(*p<0.05; **p<0.01; ***p<0.001; n.a..= not analysed).

C1 inactivator reduced complement activation but had no detectable effect on the coagulation parameters. Soluble heparin, however, prevented clotting and there was a remarkable improvement with respect to platelet count and generation of TAT. Notwithstanding the results obtained by the use of C1-INA, it is obvious that it should be beneficial to combine an anticoagulant with an inhibitor of complement.

Example 2: Effect of platelet inhibitor

Platelets in platelet rich plasma (PRP) and gel filtered platelets were tested in an aggregometer. Islets were added to PRP and thereafter analysed in the aggregometer. It was shown that the islets induced aggregation of the platelets (Fig. 1) and that platelets number in the sample were reduced from 375×10^9 to 236×10^9. If purified platelets without plasma proteins were used in combination with islets no aggregation and reduction in the platelet count were observed. In attempts to identify the mechanism behind the induced aggregation, an RGDS tetrapeptide to inhibit integrin binding and a monoclonal antibody against Fc recpetors on platelets were used. Addition of the RGDS peptide totally abolished the aggregation and the consumption of platelets when islets were added to PRP (Fig. 2). A similar finding was obtained if the anti-Fc receptor antibody was added (Fig. 3).
Conclusion: The experiments show that islets bind to platelets when added to PRP. This binding induce activation and aggregation of the platelets.

Example 3: Effect of surface modification of islets using a heparin conjugate

Using Corline Heparin Conjugate (c.f. WO 93/05793) containing approximately sixty mol of heparin covalently bound to one mol of straight-chained carrier, adult porcine islets were modified by irreversible adsorption of the heparin conjugate onto the surface of the islets. This was

accomplished by incubating the islets for 30 minutes at 37°C in a buffered saline solution containing heparin conjugate.

The presence of heparin at the surfaces of the islets was demonstrated by an ELISA assay for islet surface associated antithrombin (AT). Unmodified and heparin modified islets were incubated in human plasma for thirty minutes and then rinsed several times by changing buffer. The islet were then incubated with anti-AT that had been labelled with biotin. Using HRP-labelled streptavidin the uptake of anti-AT could be semiquantitatively estimated. The uptake of anti-AT on the heparin modified islets was three times higher than that on the unmodified islets showing that biologically active heparin was present on the surface of the islets. Testing of heparin modified islets in the tubing loop model resulted in less clotting compared to unmodified islets.

The present invention is expected to significantly improve the situation for IDDM patients. By administering an anticoagulant and/or inhibitor of platelet activation, optionally in combination with surface modification of islets, and optionally together with a complement inhibitor, in association with transplantation of insulin producing cells it is expected that the need of providing these patients with injections of insulin will be substantially decreased or even eliminated.

CLAIMS

1. Use of a clotting preventing agent in the production of a drug for administration in connection with transplantation of insulin producing cells in the form of isolated islets to patients with insulin dependent diabetes mellitus, IDDM.

2. Use according to claim 1, wherein the preventing agent is an anticoagulant.

3. Use according to claim 2, wherein the anticoagulant is heparin or fractions or derivatives thereof.

4. Use according to claim 3, wherein islet cells are coated with heparin or fractions or derivatives thereof by preincubation of islets in a solution containing heparin or fractions or derivatives thereof.

5. Use according to claim 1, wherein the preventing agent is an inhibitor of platelet activation.

6. Use according to claim 5, wherein the preventing agent is a RGD containing peptide or a monoclonal antibody which inhibits the interaction of platelet integrins with their specific ligands

7. Use according to claim 5, wherein the preventing agent is a monoclonal antibody or a peptide directed against the Fc receptor on platelets.

8. Use according to any of the above claims, wherein more than one preventing agent is used.

9. Use according to any of the above claims, wherein the preventing agent(s) is/are supplemented by an inhibitor of complement.

10. Isolated cells comprising islets of Langerhans, characterized by being coated with a heparin conjugate on the islet surface.

11. A method for increasing survival of islet cells in connection with transplantation of insulin producing cells to patients with insulin dependent diabetes mellitus, IDDM, comprising prevention of clotting, monitored as reduced generation of thrombin-antitrombin complex.

Fig. 1

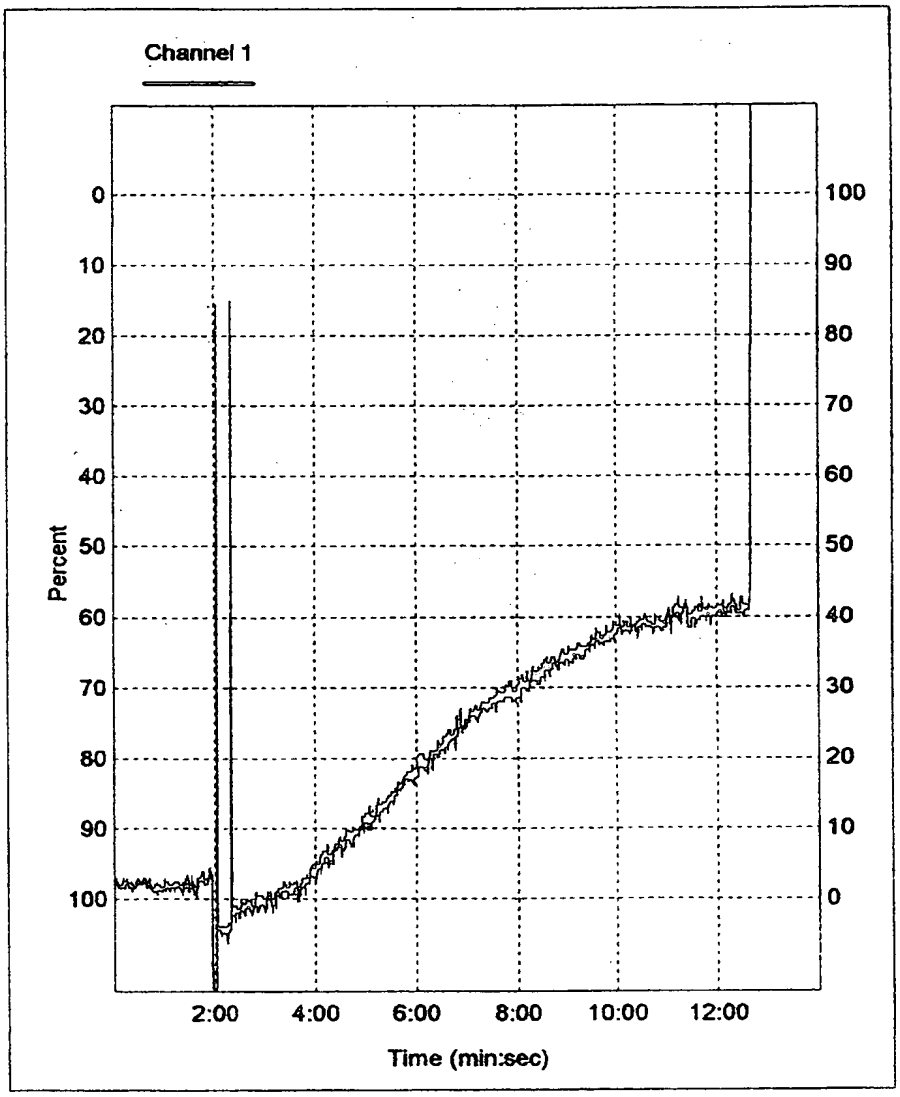
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Fig. 2

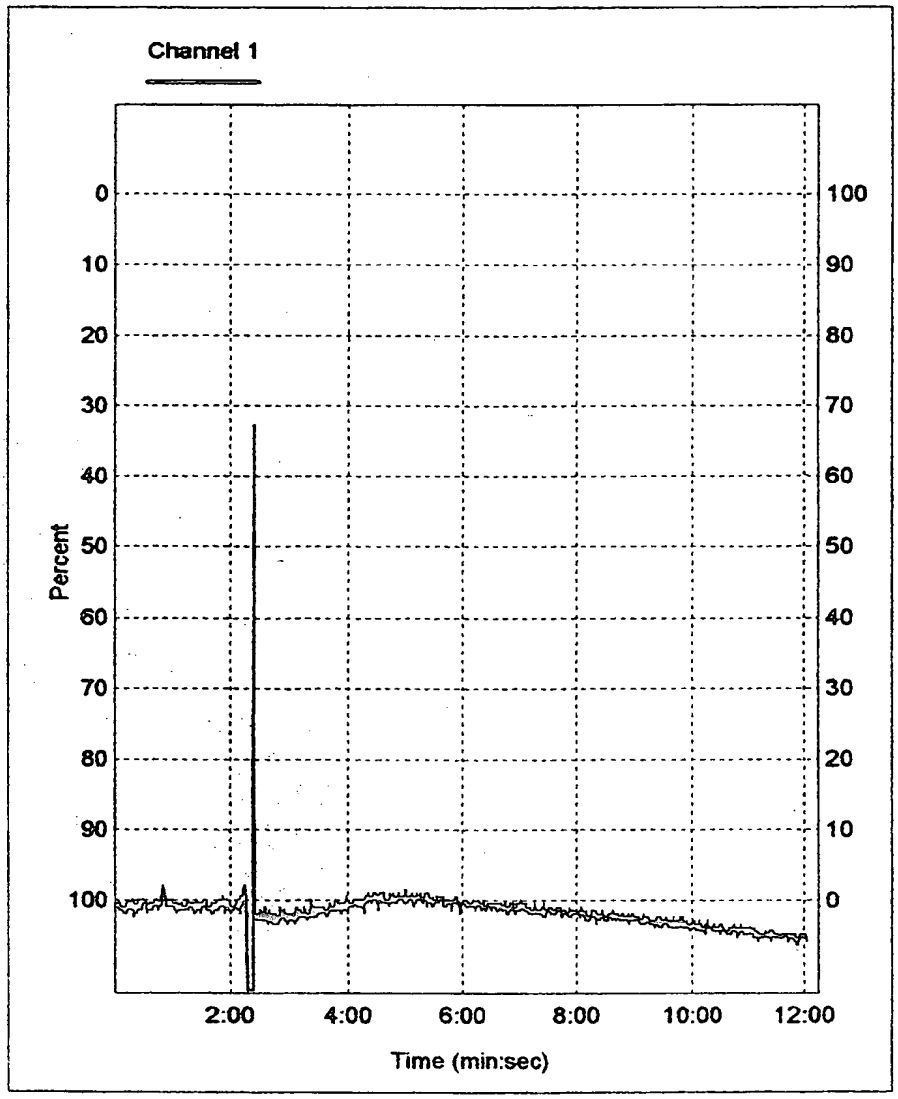
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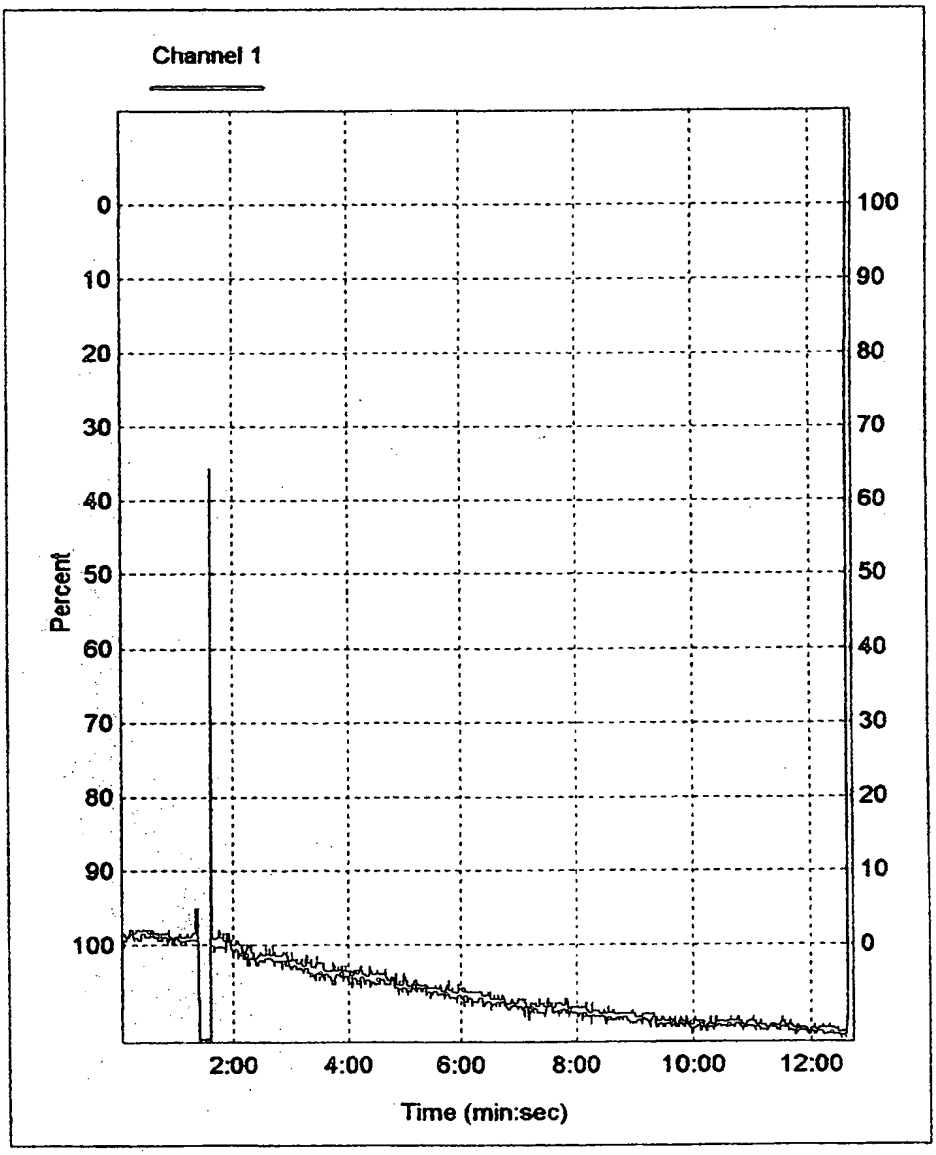
Fig. 3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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00223

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/36, A61K 38/55, C12N 5/06 // C07K 14/745
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 19623440 A1 (WAGNER, KARL-HEINZ, DR. MED.), 18 December 1997 (18.12.97) --	1-11
X	Transplantation Proceedings, Volume 28, No 3, June 1996, Y. Nomura, S. Ito, "Unpurified Islet Cell Transplantation in Diabetic Rats" page 1849 - page 1850 --	1-11
X	Transplantation Proceedings, Volume 25, No 4, August 1993, U.T. Hopt et al, "Prevention of Early Postoperative Graft Thrombosis in Pancreatic Transplantation" page 2607 - page 2608	1-8,10-11
Y	--	9

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	08 -06- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00223

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Transplantation Proceedings, Volume 20, No 3, June 1988, J. Tollemar et al, "Anticoagulation Therapyfor Prevention of Pancreatic Graft Thromb osis: Benefits and Risks" page 479 - page 480	1-8,10-11
Y		9
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X	Dialog Information Services, File 73, EMBASE, Dialog accession no. 04002091, Embase accession no. 1989171087, Rigotti P. et al: "Use of defibrotide in preventing vascular thrombosis in experimental pancreas transplantation"; & Surgical Research Communications (SURG. RES. COMMUN.) (United Kingdom) 1989, 6/2 (123-130)	1-8,10-11
Y		9
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Y	WO 9105855 A1 (IMUTRAN LIMITED), 2 May 1991 (02.05.91)	9
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X	Dialog Information Services, File 155,MEDLINE, Dialog accession no. 08102103, Medline accession no. 95134104, Tatarkiewicz K et al: "In vitro and in vivo evalutation of protamine-heparin membrane for microencapsulation of rat Langerhans islets"; & Artif Organs (UNITED STATES) Oct 1994, 18 (10) p736-9, ISSN 0160-564X	10
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/00223

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **11**
because they relate to subject matter not required to be searched by this Authority, namely:

see next sheet

2. ☒ Claims Nos.: **1, 5, 9**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/00223

Box I.1

Claim 11 relates to a method of treatment of the human or animal body by therapy (Rule 39.1(iv)). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compound.

Box I.2

The wordings "a clotting preventing agent" of claim 1, "an inhibitor of platelet activation" of claim 5 and "an inhibitor of complement" of claim 9, earch covers an unknown number of structurally different compounds in addition to those mentioned in the application. It is not considered to be clear which compounds that are intendeded to be covered by the definitions and probably the number of compounds are too many to be able to permit a search for the whole scope of the claims. Therefore, the international search has been incomplete and restricted to those compounds exemplified in the application.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 00/00223

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19623440 A1	18/12/97	NONE	
WO 9105855 A1	02/05/91	AT 139562 T	15/07/96
		AU 654116 B	27/10/94
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		DE 495852 T	31/03/94
		DE 69027535 D,T	28/11/96
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		SE 0495852 T3	
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		IE 75345 B	27/08/97
		IL 95970 D	00/00/00
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		JP 5503074 T	27/05/93
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		RO 109863 A	30/06/95
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(54) Title: A NOVEL CONJUGATE, ITS PREPARATION AND USE AND A SUBSTRATE PREPARED WITH THE CONJUGATE

(57) Abstract

The invention relates to a substantially water-soluble biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans in a non-active part thereof are anchored through covalent bonds. The invention also relates to the production of the conjugate, the preparation of a substrate surface with the conjugate, a substrate surface thus prepared, and the conjugate for use as a therapeutical agent.

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A NOVEL CONJUGATE, ITS PREPARATI N AND USE AND A SUBSTRATE PREPARED WITH THE CONJUGATE

The present invention relates to a novel biologically active conjugate based upon sulphated glycosaminoglycan, a process of preparing the conjugate, a substrate whose surface has been prepared with such a conjugate, and a process of surface-preparation using the conjugate.

Sulphated glycosaminoglycans is the common name of a number of endogenous sulphated mucopolysaccharides, such as e.g. heparin, heparan sulphate, dermatan sulphate and chondroitin sulphate, which exhibit a number of different biological properties. While the invention relates to sulphated glycosaminoglycans generally, it will, however, in the following to a great extent be described with regard to the glycosaminoglycan that has so far found the greatest medical use, viz. heparin.

Heparin occurs naturally complex-bound to protein in various mammalian tissues, such as the intestine, liver and lung, as well as in mast cells, and has then a molecular weight which may extend up to 100,000, while commercially available preparations have a molecular weight varying between about 6,000 and 20,000 depending on the source and the determination method. It consists of alternating glucuronic acid and glucosamine units, and the anti-coagulating effect has been shown to be linked to a specific pentasaccharide unit of the molecule which has antithrombin-binding properties.

Due to its anti-coagulating properties, heparin, which is usually prepared from intestinal mucosa from pig, has found use as an agent for dissolving thromboses, but perhaps above all for preventing the formation thereof. The latter is the case for inter alia procedures in, for example, the treatment of renal disease, open cardiac surg ry and intensive care, which pr cedures involve treating th patient's blood in a circulating system outside the body, so-called extra-corporeal circulation (e.g. artificial kidneys, heart-lung machines,

oxygenators), where the blood will get in contact with various materials for ign to the body.

To eliminate the clotting ability of the blood in such systems, and thereby avoid clogging by blood clots, high doses of heparin must be added to the blood. Due to the accompanying substantially increased risk of bleeding, which in the worst case may lead to life-threatening conditions, efforts have for a long time past aimed at trying to instead achieve the desired coagulation preventing effect by modifying the body-foreign material with which the blood will get in contact by surface-binding heparin thereto. Decisive factors which have stimulated this development are that the structure-activity relation of heparin has been elucidated, and that a heparin-like activity has been detected on the natural vascular wall. Thus, during the last few years several reports on successful extra-corporeal treatment with systems provided with surface-bound heparin have been published.

The surface-modification with heparin has, however, not been restricted the above mentioned contexts with extra-corporeal blood circulation, but has also come to be regarded as a more general solution to the problem of achieving biocompatibility of various devices within medical care which get in contact with blood and other body tissues. For example, surface-heparinization has also been used to improve the biocompatibility of intraocular lenses.

The hitherto used technical solutions to the problem of immobilizing heparin may be divided into two main principles, ionically and covalently bound heparin, which will be described in more detail in the following. To accomplish a surface which exhibits the desired biocompatibility based on immobilized heparin, it is important that heparin is immobilized such that its biological activity is maintained. As mentioned in the introductory part, th biological activity of heparin resides in a sp cific antithrombin-binding pentasaccharide structure which must remain intact after the immobilization on the surface to be capable of interacting with the con-

stituents of the blood. In th majority of scientific articles and patents related to immobilization of heparin, in particular those published earlier than 1980, this aspect has not been satisfied, and much less have results been presented which makes it possible to judge whether the preparation method will lead to a perfect biocompatible surface. Hereinafter a review of previously known methods of immobilizing heparin is given.

I. IONICALLY BOUND HEPARIN

Since heparin contains a great number of negatively charged groups, the heparin molecule is capable of binding relatively strongly to cationic surfaces through only electrostatic interaction. A common procedure consists in precipitating heparin from an aqueous solution thereof with a cationic surfactant, and subsequently dissolving the dried precipitate with an organic solvent. The latter solvent is then used for a so-called dip-dry procedure. Various branched surfactants have been tested in order to reduce the release rate. Other methods are based upon adsorption of heparin to quaternary ammonium groups. A great disadvantage which the ionic bound heparin surfaces have in common is that their stability with regard to the release of heparin in contact with blood is insufficient.

O. Larm et al. describes in Biomat., Med. Dev., Art. Org., 11 (1983) 161-173 inter alia a method of preparing a stable ionically bound surface. The bound heparin is, however, reported to have lost its biological activity, which may be related to the fact that each individual heparin molecule was bound too strongly such that the anti-thrombin binding sequence could not interact with circulating components in blood.

A stabilizing treatment of an ionically bound heparin complex with glutaraldehyde has been described in US-A-3,810,781 and US-A-4,118,485. As appears from scientific reports, these preparation alternatives do not lead to compl tely stabl surfaces. Thus, heparin and probably various reaction products with glutaraldehyde are released to the blood path during the initial c ntact phase.

II. COVALENTLY BOUND HEPARIN

From a purely chemical viewpoint there are a number of different ways of immobilizing heparin with covalent bonds. With cyanogen bromide, carbodiimide and similar generally used coupling reagents there is, however, an obvious risk that each heparin molecule will be bound by several bonds including bonds in the active sequence, and that the heparin thereby will lose its biological activity. Covalent coupling reagents are besides always toxic as such and should therefore not get into contact with the final product.

US-A-4,613,665 describes, however, a method of coupling heparin and other polysaccharides via a single reactive aldehyde group located terminally in the heparin molecule. Hereby heparin may be bound covalently without the antithrombin-binding sequence being involved in coupling. However, the method requires that heparin is partially degraded and that the strongly toxic substance cyanoborohydride is present in the final preparation step.

EP-A-351,314 describes a method of coupling heparin to substrate surfaces containing free amino groups (through treatment of the surface with e.g. polyethylenimine or chitosan) by utilizing free amino groups of heparin which have been subjected to N-desulphation. Cross-linking is then performed with polyfunctional aldehydes, such as e.g. glutaraldehyde. The reaction step with glutaraldehyde can, however, not be controlled with certainty to prevent the active sequence from being involved, and the method as such is relatively complicated to carry out from the technical point of view.

US-A-4,239,664 describes a PVP-heparin polymer prepared through modification of PVP such that the polymer contains imidoyl ions which then are reacted with hydroxyl groups on heparin. The method necessarily gives multiple non-specific bonds to the heparin, detrimentally ffecting the biological activity thereof. The PVP-h parin polymer is consistently said to have a low anti-coagulating activity.

EP-A-294 905 discloses a polymer substrate having an anticoagulant like heparin linked thereto via a polyacid. The substrate is prepared by covalently binding the polyacid to a small number of reactive groups on the polymer surface to thereby multiply the number of available surface reactive groups. The anticoagulant is then covalently coupled to carboxyl or amino groups of the polyacid, specifically by the method described in the above-mentioned US-A-4,613,665, the disadvantages of which has already been mentioned.

US-A-4,415,490 discloses a non-thrombogenic material wherein heparin is coupled to various polymers through only one acetal or hemiacetal bond at each binding site. In one embodiment, aldehyde groups are introduced into a polymer, such as cellulose, which aldehyde groups then are reacted with hydroxyl groups in heparin. This process will involve a plurality of the hydroxyl groups of each heparin molecule, and since hydroxyl groups are available in the biologically active sequence of heparin, which sequence was not actually known and described in the literature on the filing date of the patent, there is an apparent risk that also hydroxyl groups in the active sequence will be involved, resulting in the final product being inactive. In an alternative embodiment, aldehyde groups are instead introduced into the heparin by periodation. Also this embodiment lacks specificity, and binding will therefore occur randomly in the heparin chain, including the active sequence.

As will appear from the above, the methods known so far for surface-heparinization thus suffer from more or less serious disadvantages. There is therefore a need for a method of surface-heparinization which is simple to perform and which provides a stable heparinized surface free from toxic substances and with retained biological activity of the heparin.

Also the use of heparin as a th rapeutical agent has limitations due to the short half-life and/or affinity of h parin. This is particularly th case for the use of

heparin as an anticoagulant, but also, for example, its studied use as a growth inhibitor of smooth muscle cells in case of vessel damages (hyperplasia), as an anti-inflammatory agent for e.g. rheumatoid arthritis, and as an agent for controlling the formation of blood vessels (angiogenesis). A review of the different properties of heparin is to be found in "Heparin: Clinical and biological properties. Clinical applications." Eds: Lane and Lindahl, Edward Arnold, London, 1989. There is therefore a need of heparin preparations having a prolonged half-life and increased affinity.

According to the present invention a novel biologically active conjugate is suggested based upon sulphated glycosaminoglycans, by means of which conjugate the properties of the sulphated glycosaminoglycans may be utilized considerably more efficiently than with the individual substances. Such a conjugate may inter alia easily be made to bind stably to a substrate surface having affinity to the conjugate and may thereby, for example, in the case of heparin be used for simpler and more efficient surface-heparinization than with previous methods. Further, such a conjugate may provide glycosaminoglycan preparations having a longer half-life and an improved affinity compared with preparations based upon the pure substances.

As already mentioned for heparin, the sulphated glycosaminoglycans exist naturally bound to proteins. Thus, for example, in the case of heparin, about 15 heparin chains are bound to a protein of about 25 amino acid residues, while a proteoglycan containing heparan sulphate has fewer and considerably more sparsely arranged heparan sulphate chains. The natural conjugates are very difficult to prepare in pure form and have as far as we know not been suggested for therapeutical or similar use. The invention is based upon the concept of providing a semi- or fully-synthetic conjugate between sulphated glycosaminoglycans and a polymeric carrier, which conjugat , inter alia by containing more molecules of the glycosaminoglycan in question, has improv d properties in relation to the

individual glycosaminoglycans as well as the natural conjugates, and which conjugate further has the important advantage that the relative composition may be varied in a controllable way to suit different applications.

In its broadest scope the present invention thus provides an at least substantially water-soluble, biologically active conjugate (macromolecule), preferably in substantially pure form, comprising a substantially straight-chained organic homo- or heteropolymer having a number of functional groups distributed along the polymer backbone chain, via which groups at least about 20 molecules from the group of sulphated glycosaminoglycans (GAG) in a non-active part thereof are anchored through covalent bonds.

Such a conjugate may conceptually be described as a synthetic proteoglycan, the relative composition of which may be varied in a controllable way and adapted to the intended application.

The expression "sulphated glycosaminoglycans" herein is meant to comprise not only the substances which are normally included in the term, such as e.g. heparin, heparan sulphate, dermatan sulphate and chondroitin sulphate, but also fragments and derivatives of these substances which are functional for the purpose.

The substantially linear polymer chain which is to function as the carrier for the glycosaminoglycan residues should, of course, be substantially biologically inert after the coupling of the glycosaminoglycan or -glycans in question, in the sense that it should be devoid of at least interfering biological activity. As is readily understood, it should in order to permit coupling of a plurality of glycosaminoglycan residues be provided with a number of functional groups, such as e.g. amino, hydroxyl or carboxyl groups, distributed along the chain and capable of, after optional modification, being coupled to the glycosaminoglycan, either directly or via a coupling sequence. It is in this context to be noted that the glycosaminoglycan in question, depending on its

preparation, may still have the terminal residue of its natural conjugate protein associated thereto, and that the binding then, of course, advantageously will take place via e.g. an amino acid in such a residue.

Further, the carrier polymer should preferably have a good solubility in water. At least it should, in accordance with what has previously been said about the conjugate, be at least substantially water-soluble after the coupling of the glycosaminoglycan groups. Specific polymer chains which may be suitable for the purposes of the invention will readily be apparent to the skilled person after having taken part of the general inventive concept. This is, of course, also the case for the degree of branching on the polymer chain that may be permitted within the scope of the expression "substantially linear".

Preferably, however, the polymer chain is a natural or synthetic polypeptide, polysaccharide or aliphatic polymer. As specific examples may be mentioned polylysine, polyornithine, chitosan, polyimine and polyallylamine.

With regard to the fact that it is usually desired that the glycosaminoglycan will maintain its biological activity after the binding to the polymer carrier, it is preferred that each glycosaminoglycan molecule is bound terminally and by only a single bond to the carrier polymer. Suitably, the glycosaminoglycan is bound via an amino acid, and then preferably a terminal amino acid, but also free amino groups of a glucosamine unit may be used. The latter may exist free as such or may have been liberated through desulphation or deacetylation.

The number of glycosaminoglycan residues per polymer backbone chain is, as mentioned above, at least 20, but preferably higher, usually at least 30. Depending on the polymer backbone chain used, it may be preferred to have at least 60 and even more than 100 glycosaminoglycan residues per polymer backbone chain, as will appear from the working examples presented further on. The upper limit depends on the circumstances and is set inter alia by the solubility properti s of the selected carrier polymer, how high a

viscosity that may be permitt d, etc. In addition to th intended use of the specific conjugate, the optimum number of glycosaminoglycan units will also depend on the carrier polymer, and then particularly the size thereof. In the case of electrostatic binding of the conjugate to a substrate surface, which will be discussed in more detail below, the charge density of the substrate surface will, of course, also have to be considered. Thus, the glycosaminoglycan residues should not be located so closely that they will interfere with each other, but neither should they have too wide gaps between them. As an example it may be mentioned that e.g. polylysine as a carrier polymer should have a molecular weight higher than about 50,000. The suitable number of glycosaminoglycan residues for each specific carrier polymer and use, respectively, will, however, readily be determined by the skilled person.

Particularly in the case that an amino-functional polymer is used as the carrier, it may in some cases, especially when the polymer backbone chain is sparsely substituted with glycosaminoglycans, be favourable to block the remaining free amino groups, which, for example, may be effected by acetylation. An alternative approach might be to substitute a desired number of amino groups with e.g. methyl groups before attaching the glycosaminoglycans.

As already indicated, the novel conjugate according to the invention may be bound to a surface having affinity to the conjugate (usually but not necessarily to the glycosaminoglycan residues) so as to thereby provide the surface with the desired biological activity. According to a further aspect of the invention, such a prepared surface is accomplished by simply contacting, under suitable conditions, a biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans are anchored by covalent bonds, with a surface having affinity to the conjugate.

Another aspect of the invention provides a substrate surface having affinity-bound thereto a biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans are anchored by covalent bonds.

A preferred form of affinity between the conjugate and the substrate surface is of electrostatic nature, and more particularly that binding takes place by electrostatic interaction between the glycosaminoglycan residues and the substrate surface, as will be illustrated in more detail hereinafter.

Since the glycosaminoglycan molecules of a conjugate according to the invention are in great excess in relation to the carrier polymer, the conjugate may be regarded as a "macromolecular glycosaminoglycan". The number of anion groups per conjugate will thereby by far exceed the number existing per glycosaminoglycan molecule, which results in the conjugate, by virtue of its size, being capable of binding irreversibly to a cationic surface through ionic interaction. For the conjugate to be released from the surface, all glycosaminoglycan residues must, of course, be released from the surface simultaneously, which in contrast to the release of "free" glycosaminoglycan molecules will require a considerable supply of energy.

With the exception of certain situations which will be described further on, it is generally intended that the biological activity of the conjugate should be due to the glycosaminoglycan residues. In such a case, the number of glycosaminoglycan residues should be sufficient for a certain part of these residues per carrier polymer chain to be capable of together mediating a strong, irreversible binding to a surface which has been provided with cationic groups, while the remaining glycosaminoglycan chains freely can exert their biological activity by interacting with a biological tissue, e.g. the constituents of the blood.

Surface-preparation with a glyc saminoglycan according to the above is thus based upon a combination of covalent binding and ionic interaction, a considerable advantage being that the conjugate is prepared as an intermediate product, which means that all coupling chemistry may be made separately from the final product. Further, the final surface modification process becomes very simple and may be performed in a reproducible manner. For example, surface-heparinization with a heparin conjugate according to the invention therefore provides, as already mentioned, a considerably simplified method for efficient heparinization in relation to current methods for surface-heparinization. Notwithstanding the foregoing, it is, of course, possible to optionally carry out a cross-linking step after the affinity adsorption of the conjugate to the substrate surface in order to improve the stability of the heparinized surface even further.

A conjugate for use according to this particular aspect of the invention will thus have an electrostatic net charge which is sufficient to permit substantially irreversible binding to an oppositely charged substrate surface.

The substrate material to be surface-prepared, e.g. surface-heparinized, according to the above may in principle be any material that is desired to be made biocompatible, provided that its surface is or may be made cationic. As described previously, the invention may apply to a body-foreign material, such as various polymers, metals and ceramics. It may, however, also apply to an endogenous material, i.e. a tissue surface exhibiting affinity for the glycosaminoglycan in question. In this connection it is of interest to note that the healthy natural vessel wall in its outermost structure against the blood contains sulphated glycosaminoglycans having antithrombin-binding pentasaccharide sequences.

Various m thods for making a substrate surface cati nic are well known. Treatment with polyimine has proved to be a suitable method, but also other polyamines,

such as e.g. polylysine, chitosan or polyallylamine, may be used, as will be described in the working examples b low.

The novel glycosaminoglycan conjugate may within the scope of the invention also contain chains of one or more other substances bound to the carrier polymer in addition to the glycosaminoglycan chains, e.g. another biologically active substance. Such other biologically active substance may in that case be intended to act simultaneously with or separate from the glycosaminoglycan activity. In the latter case, only the biological activity of the complementary substance would be of interest, the glycosaminoglycans only being utilized for the affinity binding to the substrate surface. The conjugate according to the invention may thus also only function as a carrier for desired biologically active substances which one wants to bind to a surface. Examples of substances which in addition to the glycosaminoglycans may be bound to the polymer backbone chain are growth factors, enzymes, antibodies, matrix proteins, steroids, etc. It is in this context also to be noted that a conjugate with very specific adsorption properties may be accomplished, for example, with monoclonal antibodies as a complement to the glycosaminoglycan units.

Optionally, it may in the case of such combined conjugates be desired to suppress the biological activity of the glycosaminoglycan itself, which, for example, in the case of the coagulation inhibiting activity of heparin may be effected by desulphation. In such a case, the biological activity of the conjugate would thus be completely linked to the activity of complementary substances which are bound to the polymer backbone chain.

While in many cases it is the surface-binding effect of the conjugate that is essential, not to say necessary, this effect is, however, in certain cases of less interest, and it may even for some applications be desired to suppress it more or less completely. In the same way it may, as mentioned above for the combined conjugates, also in the case of a pure glycosaminoglycan conjugate be

desired to eliminate or at least reduce the biological activity of the glycosaminoglycans. In som cases, such as e.g. for heparin, the glycosaminoglycan may have several different biological effects, and depending on the intended application, one biological activity may then be suppressed in favour of another. For example, in the case of heparin the anticoagulating effect thereof may as above be inhibited by desulphation, while another biological activity which is not linked to the previously mentioned pentasaccharide unit will remain unaffected.

As appears from the above, the composition of the novel conjugate may thus be varied within wide limits to be adapted to different application fields.

Another aspect of the invention relates to the production of the described conjugate by providing a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, and to these functional groups, optionally via a coupling reagent, covalently binding a number of molecules from the group of sulphated glycosaminoglycans in a non-active part thereof. This may within the scope of the invention be performed in several different ways.

Thus, the glycosaminoglycan may, for example, be bound directly to an amino-functional polymer chain utilizing a nitrous acid degraded glycosaminoglycan having a terminally located aldehyde group prepared according to the method described in US-A-4,613,665. This method involves, however, a restriction to a partially degraded glycosaminoglycan, and the degree of substitution is difficult to control. Also, practical difficulties arise due to the fact that the polymer is easily precipitated by the glycosaminoglycan.

According to a preferred method, the glycosaminoglycan is instead bound to the polymer chain by means of a coupling reagent, and preferably a heterobifunctional one. It may be noted that bifunctional coupling reagents for e.g. hydroxyl or amino groups can, however, generally not be used, since they will lead to intra- as well as inter-

molecular cross-linking with consequential blocking and aggregation, respectively.

As an example of how a conjugate according to the invention may be prepared, the coupling of heparin to a polylysine will now briefly be described. By selecting a polylysine having a molecular weight above 400,000, a synthetic proteoglycan having up to 500 heparin chains per carrier molecule may be prepared. A hetero-bifunctional coupling reagent suitable for this purpose, N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), is coupled to amino groups on the polylysine, the SPDP-substituted polylysine then being purified chromatographically. In a separate coupling step, SPDP is also coupled to amino groups on heparin which are present either in terminal amino acid residues or as free glucosamine (the latter content may be controlled via N-desulphation or N-deacetylation). The SPDP-groups are reduced to thiol function, whereupon the SH-substituted heparin is purified chromatographically. The content of SPDP groups in polylysine and SH-groups in heparin, respectively, are determined spectrophotometrically, and heparin is mixed with polylysine in an equimolecular amount with regard to SPDP and SH, heparin being bound covalently to polylysine via disulphide exchange, the reaction rate of which may be followed spectrophotometrically. It has surprisingly been found that the precipitation reaction between polylysine and heparin does not take place when polylysine has been provided with SPDP-groups, even if only a certain portion of the amino groups of polylysine have been substituted. Nevertheless, practical experiments have shown that the disulphide exchange is quicker and proceeds to completion only at a high salt concentration (suitably 3 M NaCl). When the reaction is completed, the conjugate is purified chromatographically, free heparin and low-molecular r action products b ing rem ved.

Concerning the stability of the thus pr pared heparin conjugate in different environments, it has surprisingly been found that the obtained disulphide bridges which

couple heparin to the polymer backbone chain cannot be cleaved with glutathione but only with low-molecular non-physiological thiol reagents, such as e.g. mercaptoethanol.

It is further to be noted that a substantial advantage of heparinizing with a heparin conjugate according to the invention resides in the possibility of starting from a less processed heparin raw material than in the conventional processes.

The invention is illustrated further in the following Examples.

EXAMPLE 1

Preparation of conjugates and test of surface-bound biological activity

Two different batches of heparin (Heparin, Kabi Pharmacia AB, Sweden, molecular weight about 12,000) was used. The content of amino acids and the relative occurrence of free primary amino groups were analysed, the following results being obtained.

	Amino acid nitrogen (μg/ml)	Total nitrogen (μg/ml)	Primary amine (rel. scale)
Heparin A	0.36	5.38	5,000
Heparin B	0.08	5.37	340

Since heparin B exhibited a very low content of free amine, N-desulphation according to the method described by Yuko Inone et al., Carbohydrate Research, 46 (1976) 87-95, was performed. After carried out N-desulphation, the value 18,000 on the relative scale for primary amine was obtained.

Heparin A and heparin B (desulphated) were dissolved in phosphate buffer, pH 7.5, (200 mg/4 ml), to which 1 ml of SPDP (10 mg/ml MeOH) was added under stirring, and the reaction was allowed to proceed for 20 minutes. The SPDP-substituted heparin thus obtained was purified on Sephadex® G-25 (Pharmacia LKB Biotechnology AB, Sweden). To 100 μl of the obtained sample were added 900 μl of dithiothreitol (DTT, 10 mg/ml), and the obtained absorbance was measured spectrophotometrically at 343 nm. The substitution-degree

for heparin A was 0.21 and for heparin B (desulph.) 0.17. SPDP coupled to heparin was reduced to SH by the addition of DTT and subsequent chromatographical purification.

Polylysine having a molecular weight of 450,000 was dissolved in water (20 mg/3 ml), to which 2 ml of SPDP (10 mg/ml MeOH) were added, and the reaction was allowed to proceed under shaking for 20 minutes. Purification was performed on Sephadex® G-25 (Pharmacia LKB Biotechnology AB, Sweden) with 0.15 M NaCl as eluent. The void fraction was tested with DTT, the substitution-degree being determined as 158 SPDP-groups per molecule of polylysine.

The above prepared solutions of heparin-SH and polylysine-SPDP, respectively, were adjusted to 3 M NaCl and mixed in such proportions that a ten percent excess of SH-groups in relation to SPDP-groups was obtained, and the reaction was allowed to proceed overnight. Both preparations (heparin A and heparin B (desulph.)) had then proceeded to completion, which was determined by spectrophotometrical measurement of the release of thiopyridone at 343 nm. The preparations were purified on Sephacryl® S-500 (Pharmacia LKB Biotechnology AB, Sweden) with 0.5 M NaCl as eluent, the heparin-polylysine conjugates emerging as a void peak with baseline separation to free heparin. The content of heparin was determined with the Orcinol assay described in Larsson, R., et al., Biomaterials 10 (1989) 511-516.

The respective heparin conjugates were then diluted to 50 µg heparin/ml in citrate buffer, pH 3.8, with addition of 0.5 M NaCl. Tubings of polyethylene (PE) were surface-heparinized by treatment as follows:

1) Ammonium persulphate (1 %, 60°C, 120 min.)
2) Polyethylene imine (0.3 mg/ml, RT, 15 min.)
3) Heparin conjugate solution as above (RT, 120 min.).

The tubings were finally flushed with b rate buffer, pH 9, 2 x 10 min., and water.

The surface-heparinized tubings were tested with regard to the inhibiting capacity of thrombin according to

the following method. The tubings were first rotated with human plasma during 90 minutes, whereupon they were rinsed with sodium chloride solution. Then the tubings were incubated with a solution of thrombin (15 U/ml, 10 min., RT, under rotation) and rinsed with sodium chloride solution. Half of the tubings were then incubated with defibrinogenated plasma for 60 seconds. Surface-bound thrombin activity was measured by incubating the tubings with a chromogenic substrate for thrombin during 60 seconds, after which the reaction was stopped by the addition of citric acid. The obtained absorbance was measured at 405 nm. The following values were obtained.

	Conjugate with Heparin A	Conjugate with Heparin B (desulph.)
Uptake of thrombin (without defib. plasma)	0.639 ± 0.050	0.611 ± 0.156
Residual amount of thrombin (with defib. plasma)	0.003 ± 0.001	0.006 ± 0.001

The results indicate that both preparations provided a perfectly satisfactory effect with regard to uptake and inhibition of thrombin.

EXAMPLE 2

Preparation of conjugates with different substitution-degrees and test of surface-bound biological activity

A conjugate, called Conjugate I, was prepared as described in Example 1. The final substitution-degree of heparin per polylysine was 240:1.

Then another conjugate, called Conjugate II, was prepared. In this case the starting material was a polylysine with a higher substitution-degree of SPDP, prepared by adjustment of the pH to 8 in the polylysine solution before the addition of SPDP. The substitution-degree was determined as 633 SPDP-groups per polylysine molecule.

Heparin-SH was prepared as in Example 1 and was reacted with the highly substituted polylysine obtained

above. The reaction proceeded t 77 % conv rsion, and the substitution-degree of heparin per polylysine was therefore 490:1. Tubings of polyethylene (PE) were prepared and tested as described in Example 1, the following results being obtained.

	Conjugate I	Conjugate II
Uptake of thrombin (without defib. plasma)	0.516 ± 0.021	0.526 ± 0.031
Residual amount of thrombin (with defib. plasma)	0.011 ± 0.001	0.008 ± 0.001

The results indicate that both conjugates provided satisfactory results.

EXAMPLE 3

Test of surface-heparinized extra-corporeal system

An extra-corporeal system composed of the following components was used: Drainage catheter (polyvinyl chloride (PVC)), arterial cannula (PVC + steel), tubing set (PVC), pump bladder (ethylbutyl acrylate), valves (polypropylene (PP) + PE), oxygenator (polycarbonate + hollow-fibres of PP).

All the components were surface-heparinized by treatment in three steps:

1) Ammonium persulphate (1 %, 60°C, 120 min.)
2) Polyethylene imine (0.3 mg/ml, borate buffer, pH 9, RT, 15 min.)
3) Heparin-polylysine conjugate, prepared according to Example 1, was diluted to 30 µg/ml in citrate buffer, pH 3.8, with 0.5 M NaCl, and was treated at room temperature for 120 minutes. The components were finally rinsed 2 x 15 min. with borate buffer, pH 9, and water. After drying, sterilization with ethylene oxide was carried out.

The extra-corporeal system was connected to an anaesthetized pig which had not received any anti-coagulant therapy for partial by-pass between the right atrium and the aorta. The external system continuously pumped about

three litres per minute during twenty-four hours without any problems with clogging due to coagulation. The coagulation time was all the time at a constant value, indicating that there was no release of heparin to the blood path.

The results demonstrate that a complete system for extra-corporeal support circulation easily may be surface-heparinized with the heparin conjugate to obtain a stable, well-functioning heparin surface, which may be sterilized with ethylene oxide.

EXAMPLE 4

Test of biological activity of various heparin-conjugates in solution

Heparin-polylysine conjugates with different substitution-degrees were prepared according to Examples 1 and 2. The biological activity of the conjugates was determined with regard to their capability of inhibiting Factor Xa and thrombin in buffer solution containing anti-thrombin or in plasma. The results obtained were compared with corresponding standard graphs obtained by the addition of known amounts of heparin having a known specific biological activity (180 I.U./mg). The following results were obtained.

		Biological activity I.U./mg heparin			
Conjugate	Heparin/ polylysine	Xa/AT	Xa/Plasma	Tr./AT	Tr./Plasma
I	235	116	95	48	45
II	490	61	29	10	20
III	550	10	43	18	25

The results indicate that the described process may be utilized to prepare conjugates having a high as well as a low biological activity.

EXAMPLE 5

Effect of different polylysine sizes

Five different batches of polylysine with the respective mol cular weights 13,000, 64,000, 98,000, 249,000 and 464,000 were modified with SPDP in accordance

with Example 1, the following substitution-degrees (SPDP-groups per molecule of polylysine) being obtained:

Sample	Molecular weight	Substitution-degree
I	13,000	6
II	64,000	31
III	64,000	45
IV	98,000	35
V	249,000	87
VI	464,000	158

Heparin, having the value of 7,000 on the relative scale for primary amine, was modified with SPDP for the introduction of free thiol groups in accordance with Example 1, substitution-degrees of 0.2 - 0.3 being obtained. Respective conjugates were prepared according to Example 1. Separation was performed on a column with Sephacryl® S-300 or Sephacryl® S-400 (Pharmacia LKB Biotechnology AB, Sweden) as the separation medium. Conjugate I could not be separated from free heparin. For the other conjugates satisfactory separation was obtained, and the conjugates obtained could be used for surface-heparinizing tubings according to Example 1. Testing with regard to uptake and inhibition of thrombin (according to Example 1) gave the following results:

Conjugate	Uptake of thrombin (without def. plasma)	Residual amount of thrombin (with def. plasma)
I	----------	-----
II	0.012 ± 0.006	0
III	0.086 ± 0.047	0
IV	0.494 ± 0.009	0.003
V	0.532 ± 0.043	0.005
VI	0.490 ± 0.004	0.004

The results indicate that conjugates II - VI may be used according to the invention to prepare surfaces with h parin activity. However, conjugates IV - VI give the best results.

EXAMPLE 6

Preparation of conjugate with chitosan as carrier substance

Chitosan (SeaCure 110L, viscosity <20 mPas, molecular weight about 120,000, Protan Biopolymer A/S, Drammen, Norway) was dissolved to 10 mg/ml in water with 1 % acetic acid. To 1.5 ml solution was added 1.0 ml SPDP (10 mg/ml MeOH) under stirring at 50°C, and the reaction was allowed to proceed for one hour. The sample was loaded on a PD-10 column (Pharmacia LKB Biotechnology AB, Sweden) and eluted with 0.5 M NaCl with 1 % acetic acid. The void fraction was collected and analysed for presence of SPDP. The content of SPDP was determined as 0.972 μmole/ml, corresponding to about 40 SPDP-groups per chitosan molecule.

Heparin was prepared with free thiol groups according to Example 1. To the obtained heparin solution was then added sodium chloride to a final concentration of 3.5 M. The heparin solution was then added to the first prepared chitosan-SPDP solution under vigorous stirring, and the reaction was allowed to proceed at room temperature overnight. Spectrophotometrical control indicated that the reaction had proceeded to 100 %. The solution was fractionated on Sephacryl® S-300 (Pharmacia LKB Biotechnology AB, Sweden) and the void fraction was collected. Tubings of Pebax® (polyether block amide from Atochemie, France) were prepared for thrombin test according to Example 1. The following results were obtained:

Uptake of thrombin (without def. plasma)	Residual amount of thrombin (with def. plasma)
0.491 ± 0.016	0.002

The results demonstrate that a surface prepared with a chitosan-heparin conjugate gives a perfectly satisfactory effect.

EXAMPLE 7

Preparation of conjugates with polyallylamine

Polyallylamine hydrochloride (Aldrich, molecular weight about 50,000), 10 mg, was dissolved in 1.5 ml of borate buffer, pH 9, to which 1.0 ml SPDP (10 mg/ml MeOH)

was added under stirring and reacted for 30 minutes. The solution was loaded on a PD-10 column, which was eluted with 0.9 % NaCl. The void fraction was collected, and analysis indicated that it contained 8.46 µmole/ml SPDP, corresponding to about 192 SPDP-groups per molecule of polyallylamine. This product was used below for the preparation of Conjugate I.

Another 10 mg of polyallylamine hydrochloride, dissolved in water instead of borate buffer, were SPDP-substituted in the same way as above. The void fraction then contained 1.56 µmole SPDP/ml, corresponding to 32 SPDP-groups per molecule of polyallylamine. This product was then used for the preparation of Conjugate II below.

Another preparation utilized polyallylamine hydrochloride which had been partially methylated by reacting 2 µmole of polyallylamine dissolved in 7 ml of water with the pH adjusted to 3.5 with 861 µmole of formaldehyde in the presence of 1610 µmole of cyanoborohydride. After reaction over night, the modified polyallylamine was purified on Sephadex® G-25 (Pharmacia LKB Biotechnology AB, Sweden). Modified polyallylamine hydrochloride, 10 mg, was dissolved in borate buffer, pH 8, and was substituted with SPDP as described above. The void fraction then contained 1.5 µmole/ml, corresponding to about 50 SPDP groups per molecule of polyallylamine. This product was then used for the preparation of Conjugate III below.

Heparin was prepared with free thiol groups according to Example 1, whereupon the resulting heparin-SH was mixed with the respective polyallylamine-SPDP products in an equimolecular relation with regard to SH- and SPDP-groups. After reaction for one hour the salt content was raised to 3 M, and the reaction was allowed to proceed overnight at room temperature. The reaction yield was more than 95 % for all three reactions. The respective reaction solutions of Conjugates I and II were adjusted with 10 M sodium hydroxide to pH 10, and 100 µl of acetic anhydride were then added under vigorous stirring in order to acetylate

the remaining amino groups. The heparin conjugates obtained, Conjugate I, Conjugate II and Conjugate III, respectively, were purified on a Sephacryl® S-400 column (Pharmacia LKB Biotechnology AB, Sweden), the conjugates being obtained in the void fraction.

Tubings of polyethylene were prepared with the three obtained heparin conjugates for the thrombin test according to Example 1, the following results being obtained.

	Uptake of thrombin (without defib. plasma)	Res. amount of thrombin (with defib. plasma)
Conjugate I	0.437 ± 0.008	0.007 ± 0.002
Conjugate II	0.445 ± 0.020	0.003 ± 0.001
Conjugate III	0.501 ± 0.032	0.005 ± 0.002

The results demonstrate that all three conjugates give a perfectly satisfactory effect.

EXAMPLE 8

Preparation of surfaces with different amino-functional substrate surfaces

Tubings of polyethylene were heparinized as follows, the added designations A, B, C and D, respectively, indicating alternative amino-functionalizing treatments of the tubing surface:

1. Ammonium persulphate (1 %, 60°C, 60 min.)
2A. Polyethylenimine (0.3 mg/ml, borate pH 9, RT, 15 min.)
2B. Polyallylamine (10 mg/ml, borate pH 9, RT, 15 min.)
2C. Chitosan (10 mg/ml, 1 % HAc, RT, 15 min.)
2D. Polylysine (5 mg/ml in water, RT, 15 min.)
3. Heparin-polylysine conjugate prepared according to Example 1 (50 µg/ml in citrate buffer, 0.5 M NaCl, pH 3.8, RT, 120 min.).

The surfaces thus prepared were thoroughly rinsed with borate buffer, pH 9, and water.

Polyethylene tubings heparinized according to the above four alternatives were tested with regard to uptake and inhibition of thrombin, as described in Example 1, all the alt rnatives exhibiting a perfectly satisfactory effect.

EXAMPLE 9

Surface-heparinization of a lens (PMMA) and test of platelet adhesion

Intraocular lenses of polymethyl methacrylate (PMMA) were heparinized according to Example 1 and then tested with regard to adhesion of platelets.

Non-modified and surface-heparinized lenses, respectively, were incubated in fresh human citrated whole blood under constant motion for 60 minutes. The lenses were then rinsed repeatedly in sodium chloride solution to remove all adhering blood. Finally, adenosine triphosphate (ATP) was extracted from any platelets which had adhered to the lens surface, and the content of ATP obtained was determined by bioluminescence. Platelet adhesion to the heparinized lenses was reduced with 98 % compared with the untreated control lenses.

EXAMPLE 10

Adsorption of heparin conjugate to a " biological surface"

In order to examine whether a heparin conjugate prepared according to the present invention can be adsorbed irreversibly to a surface coated with thrombotic biological material the following experiments were carried out:

Non-surface-modified tubings of polyethylene were half-filled with citrated whole blood and rotated for 60 minutes. The tubings were then drained of blood and thoroughly rinsed with sodium chloride solution. Hereby the tubings were coated with thrombotic material consisting of plasma proteins and platelets in different activation stages. Heparin-polylysine conjugate prepared according to Example 1 was diluted to a final concentration of 100 µg/ml in sodium chloride solution, which solution then was rotated in the tubings for 60 minutes. The tubings were finally thoroughly rinsed with borate buffer, pH 9, and water.

The tubings thus heparinized were tested with regard to uptake and inhibition of thrombin according to Example 1, the test tubings exhibiting a perfectly satisfactory effect.

EXAMPLE 11

Combination preparation with urease

Polylysine (10 mg, molecular weight 464,000) was dissolved in 1.5 ml water, to which 1.0 ml of SPDP (10 mg/ml MeOH) was added under shaking, and the reaction was then allowed to proceed for 30 minutes. The sample was loaded on a PD-10 column and eluted with 0.9 % NaCl. The void fraction was collected, and analysis indicated that the content of SPDP was 1.053 µmole/ml.

Urease (U-1500, from jackbean, Sigma, USA) was dissolved to 10 mg/ml in phosphate buffer, pH 7.5, and filtrated through a 0.22 µm filter. The content of free SH-groups was determined as 0.161 µmole/ml.

Polylysine-SPDP dissolved in 3 M NaCl was mixed with urease, such that about 10 % of available SPDP-groups could undergo disulphide exchange with SH-groups of the urease. Spectrophotometrical measurement at 343 nm confirmed that this had taken place. Then, heparin modified with free SH-groups according to Example 1 was added, the amount of added heparin-SH corresponding to the remaining 90 % of available SPDP-groups. The reaction proceeded to completion. The obtained conjugate was finally purified on a Sephacryl® S-400 column (Pharmacia LKB Biotechnology AB, Sweden), the conjugate being obtained in the void fraction. Testing of the obtained conjugate indicated that heparin activity as well as urease activity could be detected.

CLAIMS

1. A substantially water-soluble biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups at least about 20 molecules from the group of sulphated glycosaminoglycans in a non-active part thereof are anchored through covalent bonds.

2. A conjugate according to Claim 1, characterized in that said polymer is derived from a natural or a synthetic polypeptide, polysaccharide or aliphatic polymer.

3. A conjugate according to Claim 2, characterized in that said polymer chain is derived from polylysine, polyornithine, chitosan, polyimine or polyallylamine.

4. A conjugate according to Claim 1, 2 or 3, characterized in that the glycosaminoglycans substantially are bound to the polymer backbone chain via a single bond, preferably terminally.

5. A conjugate according to any one of Claims 1 to 4, characterized in that the glycosaminoglycans are bound to the polymer backbone chain via an amino group associated with the glycosaminoglycans.

6. A conjugate according to any one of Claims 1 to 5, characterized in that the conjugate due to the glycosaminoglycans has a sufficient polyanionic character when dissolved in water to be capable of being bound substantially irreversibly along substantially the whole of its length to a positively charged substrate surface through electrostatic interaction.

7. A conjugate according to any one of Claims 1 to 6, characterized in that it has at least 30 glycosaminoglycan residues.

8. A conjugate according to Claim 7, characterized in that it has at least 100 glycosaminoglycan residues.

9. A conjugate according to any one of Claims 1 to 8, characterized in that said glycosaminoglycan is heparin or a fragment or derivative thereof.

10. A conjugate according to any one of Claims 1 to 9, characterized in that the glycosaminoglycan residues are bound to the polymer backbone chain via a coupling sequence.

11. A conjugate according to Claim 10, characterized in that said coupling sequence is derived from a hetero-bifunctional coupling reagent.

12 A conjugate according to any one of Claims 1 to 11, characterized in that the polymer backbone chain in addition to the glycosaminoglycans supports residues of at least one additional, biologically active substance.

13. A prepared substrate surface comprising a biologically active conjugate affinity-bound to the surface, characterized in that the conjugate comprises a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans are anchored through covalent bonds, the conjugate preferably being bound to the surface through electrostatic interaction between the conjugate and the substrate surface.

14. A prepared substrate surface according to Claim 13, characterized in that the biol gically active conjugate is a conjugate according to any one of Claims 1 to 11.

15. A process of preparing a biologically active conjugate comprising a substantially straight-chained organic polymer supporting a number of molecules from the group of sulphated glycosaminoglycans, characterized in that the process comprises providing a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, and to these functional groups, optionally via a coupling reagent, covalently binding a number of molecules from the group of sulphated glycosaminoglycans in a non-active part thereof.

16. A process of preparing a surface with sulphated glycosaminoglycans, characterized by contacting a conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans are anchored through covalent bonds, with a substrate surface having affinity to the conjugate, such that the conjugate is bound substantially irreversibly thereto.

17. A process according to Claim 16, characterized in that the conjugate has polyanionic character and that the substrate surface is cationic.

18. A biologically active conjugate according to any one of Claims 1 to 12 for use as a therapeutical agent.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00672

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 31/725, 47/48, A 61 L 33/00, C 08 B 37/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A1, 0294905 (SENTRON V.O.F.) 14 December 1988, see the whole document --	1-3
X	US, A, 4415490 (YASUSHI JOH) 15 November 1983, see the whole document --	1-3
A	WO, A1, 8700060 (BATTELLE MEMORIAL INSTITUTE) 15 January 1987, see the whole document --	1-17
A	EP, A1, 0212933 (KOKEN CO. LTD) 4 March 1987, see the whole document --	1-17

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* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd December 1992	29 -12- 1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Anneli Jönsson

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 92/00672

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 02/12/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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